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Thermophilin 13, a Nontypical Antilisterial Poration Complex Bacteriocin, That Functions without a Receptor*

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A novel broad host range antimicrobial substance, Thermophilin 13, has been isolated and purified from the growth medium of *Streptococcus thermophilus*. Thermophilin 13 is composed of the antibacterial peptide ThmA (M_r of 5776) and the enhancing factor ThmB (M_r of 3910); the latter peptide increased the activity of ThmA ~40 ×. Both peptides are encoded by a single operon, and an equimolar ratio was optimal for Thermophilin 13 activity. Despite the antilisterial activity of Thermophilin 13, neither ThmA nor ThmB contain the YGNGV-C consensus sequence of *Listeria*-active peptides, and post-translational modifications comparable to that in the lantibiotics are also absent. Mass spectrometry did reveal the apparent oxidation of methionines in ThmA, which resulted in a peptide that could not be enhanced any longer by ThmB, whereas the intrinsic bactericidal activity was normal. Thermophilin 13 dissipated the membrane potential and the pH gradient in liposomes, and this activity was independent of membrane components from a sensitive strain (*e.g.* lipid or proteinaceous receptor). Models of possible poration complexes formed are proposed on the basis of sequence comparisons, structure predictions, and the functional analysis of Thermophilin 13.

Antibacterial membrane-acting peptides form a heterogeneous family of structures that can be subdivided in different classes on the basis of primary sequence, mode of synthesis (ribosomal *versus* non-ribosomal), post-translational modifications, and structure (linear, cyclic, α-helical, and β-sheet). The following classes can be discriminated: (i) antibiotics (non-ribosomal synthesis, *e.g.* gramicidins (1)); (ii) lantibiotics (lanthionine-containing peptides, *e.g.* Nisin (2)); (iii) host defense peptides (3) of mammals (*e.g.* defensins), frogs (*e.g.* magainins), and insects (*e.g.* cecropins). Some peptides have a strong hemolytic activity in addition to antibacterial properties, like (iv) bee venoms (*e.g.* melittin (4)) and (v) bacterial cytolysins (*e.g. Staphylococcus aureus* δ-toxin (5)). A particular class of antibacterial peptides is formed by (vi) the bacteriocins (*e.g.* lactococcins, Pediocins), which are produced by lactic acid bacteria and preferentially inhibit species that are closely related to the producer (6).

Besides their differences in structure and mode of membrane interaction, antibacterial membrane-acting peptides may differ in their requirement for specific lipids or proteins and/or membrane potential or pH gradient to insert properly into target membranes and/or to exhibit maximal activity. These requirements determine to a large extent the inhibitory spectrum of a particular peptide. Moreover, in case of the lantibiotics and bacteriocins, an immunity protein is synthesized that protects the producer organism (7). Bacteriocins are often considered to be different from other antibacterial membrane-acting peptides by the fact that they require a proteinaceous membrane component (*i.e.* receptor) for antibacterial activity, which is consistent with their narrow host range specificity (6). A property of some of the peptide bacteriocins is their ability to form poration complexes that are composed of two different peptides (8, 9). These bacteriocins have a very narrow inhibitory spectrum of activity (6). Finally, the so-called *Listeria*-active bacteriocins share a consensus sequence motif (YGNGV-C) at their amino terminus (9) which could be of importance for their ability to inhibit *Listeria* species. The "*Listeria*-active bacteriocins" have not been reported to form poration complexes.

In this article, we describe the properties of a broad host range pore-forming antimicrobial activity, named Thermophilin 13. The compound has structural and functional features of bacteriocins, in particular those that form poration complexes. However, none of the two peptides of Thermophilin 13 owes the antilisterial YGNGV-C consensus sequence and a proteinaceous receptor is not required for activity. We also present a model of the poration complex formed by Thermophilin 13 on the basis of structural similarities with host defense peptides and other pore-forming structures.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—The bacteriocin producer *Streptococcus thermophilus* SFi13 as well as the indicator micro-organisms are from the Nestlé strain collection. The indicator strain used to assess the bacteriocin concentration (activity) during purification was *S. thermophilus* SFi3. *Lactococcus*, *Streptococcus*, and *Enterococcus* strains were grown semi-anaerobically in M17 broth (Oxoid, U K) supplemented with 0.5% (w/v) glucose at 30, 42, and 30 °C, respectively. *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Bifidobacteria* strains were grown in MRS, 0.5% (w/v) glucose (Sanofi Diagnostics Pasteur, France) at 30 °C except for the thermophilic *Lactobacilli* (42 °C) and *Bifidobacterium* (37 °C). *Clostridium* strains (spores and vegetative cells) were grown in RCM (Oxoid) at 30 °C under an atmosphere of 85% (v/v) N_2, 5% CO_2, 10% H_2. *Bacillus* (30 °C), *Listeria* (30 °C), *Micrococcus* (30 °C), *Staphylococcus* (30 °C), *Salmonella* (37 °C), and *Escherichia coli* strains (37 °C) were all grown in BHI (Difco) at the temperatures indicated. *E. coli* BZ234 (C600 derivative allowing α-complementation; Biozentrum, University of Basel, Switzerland) was grown in Luria broth (10).

Production and Concentration of Thermophilin 13—S. thermophilus SFi13 was grown semi-anaerobically at 42 °C in 1 liter of M17 supplemented with 1% (w/v) sucrose (M17S, M17 broth (Oxoid) supplemented

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U93029.

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with 1% (w/v) sucrose) until 2 h into the stationary phase. Cells were removed by centrifugation (20,000 × *g* for 20 min), and the pH was adjusted to 1.6 by phosphoric acid. Insoluble material was removed by centrifugation (20,000 × *g* for 20 min at 4 °C), and the soluble fraction, containing the antibacterial activity, was concentrated by trichloroacetic acid precipitation (trichloroacetic acid, 10% (w/v) final). The pellet was washed twice with cold acetone (−20 °C), resuspended in 8 ml of 0.1% trifluoroacetic acid, and heated for 20 min in boiling water. Insoluble material was removed by centrifugation; the soluble sample obtained was termed trichloroacetic acid extract.

Bacteriocin Assays—The bacteriocin activity was measured in an agar well assay. Briefly, 5 ml of M17S Top-Agar (0.75% (w/v)) were mixed with 20 μl of an overnight culture of the indicator strain SFi3 and poured on top of 35 ml of M17S-Agar (1.5% (w/v)). Wells of 70 μl were made using a 3.5-mm diameter punch-holer connected to a vacuum device, and serial dilutions of the sample were assayed in different wells. Consequently, the highest dilution still showing activity defines the activity of the 70-μl aliquot in terms of arbitrary units (AU).[1] The amount of Thermophilin 13 in a given volume (*V* μl) was expressed in total units ($U = (AU/70) \times V$). The concentration of Thermophilin 13 was defined as AU/ml = AU/70 × 1000.

Purification of Thermophilin 13 and Identification of ThmA and ThmB—The trichloroacetic acid pellet of a 1-liter culture was dissolved in 8 ml of 200 mM Tris-HCl, pH 8.0, 6 M urea plus 2 M NaCl and used for purification on a 20-ml Source 15-Phe resin (15-Phe) packed into a HR16/10 column (Pharmacia Biotech Inc.). Flow rates were kept constant at 4 ml/min. The column was first washed with 100 ml of 50 mM Tris-HCl, pH 8.0, 2 M NaCl (buffer A) before applying a 60-ml linear gradient of buffer A to buffer B (50 mM Tris-HCl, pH 8.0), which was followed by 150 ml of buffer B and 100 ml of H_2O. Thermophilin 13 was eluted with 60 ml of buffer C (70% (v/v) acetonitrile, 30% (v/v) water, and 0.1% (v/v) trifluoroacetic acid). 20 ml of 100% methanol were added to the active fractions (~40 ml total), and the volume was reduced to 24 ml by rotary evaporation (approximately 50% (v/v) methanol, final concentration). This sample was applied to a 3-ml Resource RP column (RPC, Pharmacia, NL), previously equilibrated with 50% (v/v) methanol, 50% water, 0.1% trifluoroacetic acid (buffer E). A flow rate of 2 ml/min was kept throughout the procedure. After washing with 100 ml of buffer E at a flow rate of 2 ml/min, a linear gradient from buffer E to 100% acetonitrile (in 0.1% trifluoroacetic acid) was applied in 30 min. Two main peaks were characterized by electrospray-MS and re-chromatographed separately after dilution (1:1) in 0.1% trifluoroacetic acid. Both peaks were collected from two independent runs, re-analyzed by electrospray-MS, and stored at −20 °C in their elution solvent.

Mass Spectrometry—Electrospray mass spectra were recorded on a R 3010 quadrupole mass spectrometer (NERMAG, Argenteuil, France) equipped with a custom-built pneumatically assisted electrospray (ion spray) ion source. The molecular weight of the peptides is determined by measurement of multiply charged ions (11). All molecular masses quoted in this paper are average, chemical atomic masses.

Inhibitory Spectrum—Multiwell dishes (Falcon 3046) were filled with 6 ml of Agar medium per dish and 0.7 ml of Top-Agar were inoculated with 0.1–1% of an overnight culture or 10^5 spores/ml of Top-Agar. Each strain was tested with 300 AU of Thermophilin 13 which was obtained after dilution of the trichloroacetic acid extract with 100 mM potassium phosphate, pH 7.0. A control of bacteriocin activity was included for each strain using a proteinase K-treated sample (5 μg/ml at 37 °C for 20 min). Minimum inhibitory concentration values were estimated from the size of the halos obtained with the trichloroacetic acid extract; the data were calibrated on the basis of the inhibition of *S. thermophilus* SFi3 strain (1 AU = 11 nM). This method was validated for *Clostridium botulinum*, *Listeria monocytogenes*, *Lactococcus lactis*, *Bacillus cereus*, *Bacillus subtilis* and *S. thermophilus*, using purified Thermophilin 13 (equimolar ratio of ThmA and ThmB).

Protein Determination—Protein concentrations were determined by the method of Lowry *et al.* (12), with bovine serum albumin as standard, unless indicated otherwise.

DNA Preparations—General procedures for DNA isolation from *E. coli*, restriction analysis, ligation, electrophoresis, Southern analysis, and colony hybridization were carried out as described previously (10). For preparation of genomic DNA from *S. thermophilus*, cells were grown in 2 ml of M17S until the mid-log phase, treated with lysosyme (13), and resuspended in 2 ml of STE (25% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA). SDS and RNase A were added to final concentrations of 1% (w/v) and 50 μg/ml, respectively, and the mixture was incubated for 1 h at 37 °C. Subsequently, proteinase K was added to a final concentration of 100 μg/ml, and the mixture was incubated for 2 h at 55 °C. DNA fibers were spooled in 70% ethanol, solubilized overnight in TE, and extracted with chloroform (10).

Cloning of the Bacteriocin Encoding Genes—A set of degenerate oligonucleotides was deduced from the 48 amino acid amino-terminal sequence of ThmA. The forward primer corresponded to 5′GAYAT-GGGNGGNTAYGC3′ (For1) and the reverse primers corresponded to 3′GTACANCCNCGNTAACG5′ (Rev1), 3′GTACANCCNCGNTAGCG-5′(Rev2), 3′GTACANCCNCGNTATCG5′(Rev3), 3′GTGCANCCNCG-NTAACG5′(Rev4), 3′GTGCANCCNCGNTAGCG5′(Rev5), and 3′GTG-CANCCNCGNTATCG5′(Rev6). Primers and chromosomal DNA were used at final concentrations of 5 μM and 1.3 μg/ml, respectively. Other components were added as specified in the polymerase chain reaction protocol using SuperTaq polymerase of Boehringer Mannheim, and a 5-min denaturation cycle at 95 °C was followed by 30 cycles of 30 s 95 °C, 30 s 42 °C, and 30 s 72 °C; elongation was achieved by a final 5-min step at 72 °C. Polymerase chain reaction fragments of the expected size (128 bp) were eluted, ligated into plasmid pGEM-T (Promega, CH), and propagated in *E. coli* BZ234. The 128-bp fragment was used as a hybridization probe (primer extension, Boehringer Mannheim) to identify and clone a 3.8-kb *Eco*RI-*Hin*dIII genomic DNA fragment that carried the genes for ThmA and ThmB. Recombinant strains of *E. coli* BZ234 were identified by colony hybridization using the 128-bp probe on Zeta-probe membranes (Bio-Rad) (10).

Computer Analysis—Multiple sequence alignments, data base searching, and structure prediction methods were used from the GCG package (Genetics Computer Group, Inc.), PCGENE and the EMBL worldwide web servers (PredictProtein@EMBL-Heidelberg.DE). Hairpin turns were accepted when the conformational potential for each residue of the tetrapeptide to be in turn <pt> is higher than <pα> and <pβ> (14), and the probability of forming a β-turn by a tetrapeptide was $p(t)10^{-4} > 2$ (15).

Membrane Preparations—Right-side out membrane vesicles of *S. thermophilus* and their fusion to cytochrome *c* oxidase containing liposomes (COVs) were performed in 50 mM potassium phosphate, pH 7.0, as described previously (16).

Measurement of Membrane Potential (ΔΨ) and pH Gradient (ΔpH)—The membrane potential (ΔΨ) was estimated from the distribution of tetraphenylphosphonium ion (TPP^+) using an ion-selective electrode (17). COVs or hybrid membranes were diluted into 50 mM potassium phosphate, pH 6.3, plus 5 mM $MgSO_4$, (saturated with oxygen) to a final concentration of 153 nM in cytochrome *c* oxidase. A proton motive force was generated in COVs or hybrid membranes by adding the electron donor system ascorbate (10 mM), TMPD (200 μM) plus cytochrome *c* (10 μM), and oxygen (18). The ΔΨ was calculated after correction for probe binding to the membrane (19) and using a specific internal volume of 1.5 μl/mg lipids (20). The pH gradient was monitored by the fluorescent pH indicator pyranine (21). Pyranine was entrapped in the COVs by freeze/thaw/sonication as described previously (18), and external pyranine was removed by gel filtration on Sephadex G25. Pyranine-containing COVs were diluted into 50 mM potassium phosphate, pH 6.3, plus 5 mM $MgSO_4$ (saturated with oxygen), to a final concentration of 46 nM in cytochrome *c* oxidase, and ΔpH was estimated from the changes in pyranine fluorescence.

Cytochrome c Oxidase Activity—Cytochrome *c* oxidase activity was measured in liposomes by monitoring the decrease in absorbance at 550–540 nm (α-peak) (22) in the presence and absence of Thermophilin 13. The reaction was performed in 50 mM potassium phosphate, pH 6.3, plus 5 mM $MgSO_4$ (saturated with oxygen), at a final concentration of 16 nM in cytochrome *c* oxidase and in the presence of 60 nM valinomycin plus 60 nM nigericin.

RESULTS

Purification of Thermophilin 13—S. thermophilus SFi13 produced 570 AU/ml of Thermophilin 13 when grown in M17S and harvested after 2 h in the stationary phase. Clarification of the medium supernatant by phosphoric acid resulted in the removal of exo-polysaccharides as well as 99% (w/w) of proteins (Table I); the increase in activity after trichloroacetic acid precipitation could be assigned to the decrease in pH as the activ-

[1] The abbreviations used are: AU, arbitrary units; MS, mass spectrometry; RPC, reverse phase chromatography; 15-Phe, Source 15-Phe reverse phase column HR16/10; Resource-RPC, resource reverse phase column; COVs, cytochrome *c* oxidase-containing liposomes; TPP^+, tetraphenylphosphonium ion; bp, base pair(s); kb, kilobase pair(s); ORF, open reading frames.

TABLE I
Purification of Thermophilin 13

Steps	Volume	pH	Total activity	Protein amount[a]	Specific activity
	ml		*kU*	*mg*	*kU/mg*
Supernatant	1000	4.7	570	7000	0.08
Trichloroacetic acid extract	8	2.0	940	42	22.4
Source 15-Phe	40	8.0	220	3	76
Resource-RPC	6	2.0	27[b]	0.17	159

[a] According to Lowry *et al.* (15) except for the Resource-RPC, for which the absorbance at 280 nm of each purified peptide was used.
[b] Only peak fractions were considered (see text).

ity of Thermophilin 13 increases 4-fold when the pH is lowered from 8.0 to 2.0 (data not shown). Also, variations in activity throughout the purification can be ascribed to a loss of Thermophilin 13, variations in pH, or changes in aggregation states (*i.e.* solubilization) of the bacteriocin. Thermophilin 13 was purified further on a Source 15-Phe resin, which eliminated 93% of the remaining contaminants (Table I). An activity of 113 AU/ml was obtained after the Resource-RPC for the fractions eluting between 17.5 and 19 min; a much higher activity was obtained when these fractions were mixed with those eluting between 21 and 24 min. Both sets of fractions were re-chromatographed independently on the Resource-RPC and analyzed by electrospray MS (Fig. 1). A M_r of 5776 was determined for the fractions eluting between 17.5 and 19 min, and two molecular weights were found for the fractions eluting between 21 and 24 min (Fig. 1). These latter two compounds were named ThmB (M_r of 3910) and ThmB′ (M_r of 3892). As shown in Table I, the Resource-RPC eliminated 94% of the remaining contaminating proteins but resulted in a decrease in total activity from 220 to 27 kU. The apparent loss in activity is partly due to the fact that only peak fractions, which were pure by electrospray-MS criteria, were used in the calculation of the activity. It should also be stressed that the concentration of the purified peptides was estimated from their absorbance at 280 nm, using extinction coefficients of 1490 and 5500 M^{-1} cm^{-1} for Tyr and Trp, respectively (23), rather than the Lowry assay (12) which was used throughout the purification procedure.

Antibacterial Properties of ThmA and ThmB on S. thermophilus SFi3—As already suggested by the experiments presented above, fractions containing ThmB seem to enhance the activity of ThmA. The enhancing properties of ThmB are clearly shown in Fig. 2 (*left panel*), *i.e.* the diameter of inhibition of growth by ThmA is increased in the presence of ThmB (*top left panel*). The activity of 2.2 μM ThmA was 20 AU (*top left panel*), whereas the activity of 1.1 μM ThmA plus 1.1 μM ThmB was 400 AU. In other words, the activity of ThmA is enhanced 40-fold in the presence of an equimolar concentration of ThmB. The antibacterial activity at different concentrations and ratios is presented in Fig. 3. Surfaces of the inhibition zones were plotted as a function of ThmA and ThmB concentration in the range of 0 to 2.2 μM (0–155 pmol/well). The largest inhibition zones were observed at the diagonal connecting equimolar concentration of ThmA and ThmB (Fig. 3*A*), and the minimum inhibitory concentration value of Thermophilin 13 was estimated to be 11 nM for *S. thermophilus* SFi3 as indicator strain (Fig. 3*C*). In Fig. 3*B*, the antibacterial activity of Thermophilin 13 is shown at high amounts of ThmA relative to ThmB. It appeared that 1100 nM ThmA has the same activity as 27 nM of ThmA plus 27 nM ThmB (Fig. 3*B*). Fig. 3*C* shows that ThmB has no intrinsic activity even at high concentrations (2200 nM), and Fig. 3*D* shows that a large excess of ThmB over ThmA inhibits the activity of the latter peptide.

Inhibitory Spectrum of Thermophilin 13—Cells or spores were mixed with Top-Agar and poured on top of agar medium to obtain a homogeneous lawn. Prior to growth, wells were made in which diluted, neutralized trichloroacetic acid extract or purified ThmA and/or ThmB were introduced; proteinase K-treated samples were used as negative controls. The results obtained with the neutralized extract were similar to those of ThmA plus ThmB, indicating that a single bacteriocin is produced by *S. thermophilus* SFi13 under the conditions tested. Diameters of inhibition surrounding the wells were measured after overnight growth. As for other peptide bacteriocins, a narrow inhibitory spectrum was expected for Thermophilin 13 (6, 9). Thermophilin 13, however, exerted a broad host range activity among Gram-positive bacteria (Table II). Not only lactic acid bacteria were affected, but also *L. monocytogenes* and

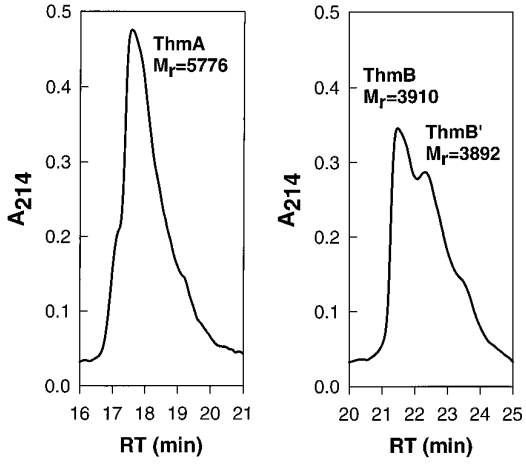

FIG. 1. **Elution profile after reverse phase chromatography (Resource-RPC).** Fractions obtained after phenyl interaction chromatography (24 ml; Source 15-Phe resin) were applied to the Resource-RPC at a flow rate of 2 ml/min in buffer A (methanol 50% (v/v), water 50%, trifluoroacetic acid 0.1%). Elution was performed with a linear gradient at 2 ml/min in 30 min from buffer A to B (100% CH_3CN, trifluoroacetic acid 0.1% (v/v)), and two peaks were re-chromatographed separately as indicated in the text; their elution profiles are presented here. Molecular weights (M_r) of the peak fractions are indicated. *RT*, retention time.

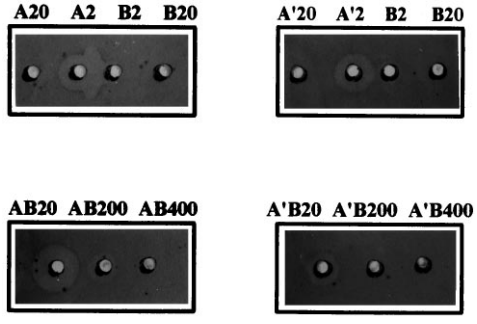

FIG. 2. **Antibacterial properties of ThmA, ThmB and ThmA′.** *Top left panel,* activity of ThmA (*A2* and *A20*) and ThmB (*B2* and *B20*) were evaluated with the agar well assay at dilutions of 2- and 20-fold. Dilution 2 corresponds to 2.2 μM, *i.e.* 154 pmol/well. *Bottom left panel,* activity of ThmA was assessed in the presence of equimolar amounts of ThmB at dilutions of 20-, 200-, and 400-fold. *Right panels,* the same experiments were performed with ThmA′ instead of ThmA.

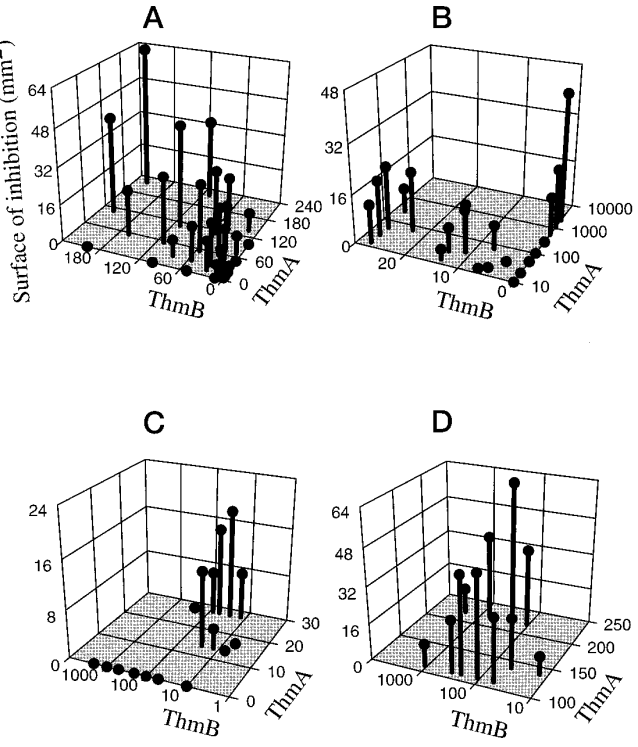

FIG. 3. **Antibacterial properties of ThmA and ThmB.** *Vertical axis,* surface inhibition in the agar well assay in mm^2; *horizontal axis,* concentrations of ThmA and ThmB expressed in nM. *A*, equimolar ratios of ThmA and ThmB; *B*, excess of ThmA; *C*, excess of ThmB; *D*, excess of ThmB at high concentrations of ThmA.

TABLE II
Inhibitory spectrum of Thermophilin 13

Species	Strains[a]	Diameter of inhibition[b]	MIC[c]
		mm	*nM*
S. thermophilus	SFi3	13.5	11
Enterococcus faecium	SFM1	10	413
Lactococcus cremoris	SC11	10	413
Lactobacillus acidophilus	LQ1	9.5	470
Lactobacillus helveticus	LH3	13.5	11
Lactobacillus fermentum	L26	10.5	206
Leuconostoc cremoris	LCC1	11	150
Leuconostoc mesenteroides	LCM18	10	413
Bifidobacterium bifidum	BB9	15.5	5
Propionibacterium	PP1	13	17
Listeria innocua	24	6.5	1885
L. monocytogenes	59	10.5	206
B. subtilis	A2	7.5	1650
B. cereus	C14	12	69
C. borulinum	100003	13	17
Clostridium tyrobutyricum	107001	9	660
Staphylococcus carnosus	STC7	8	1100
Micrococcus varians	MCV1	8	1100

[a] All strains are from the Nestlé strain collection.
[b] Diameter of inhibition observed with 300 AU of Thermophilin 13.
[c] Minimum inhibitory concentration, to observe a halo in the Agar well assay.

spore-forming micro-organisms like *C. botulinum* and *B. cereus*. Interestingly, growth from spores as well as from vegetative cells was inhibited. Bacterial genera most related to the producer (*e.g. streptococci*, *enterococci*, *lactococci*, *lactobacilli*) were inhibited to the same extent as more distantly related organisms (*e.g. bacilli*, *clostridiae*). All Gram-negative bacteria tested (*E. coli*, *Pseudomonas* and *Salmonella* species) were resistant. ThmA alone had an intrinsic activity that was enhanced by ThmB on all strains tested.

```
1621                                                          1680
TTTTTGAAGTAGTGCTATACTAGACTTGTCAAGGTTGCAACCCGACAAAATAAAAATATT
                                -35                    -10    1740
AGGTAGGAGATATTTACAATGAATACAATAACTATTTGTAAATTTGATGTTTTAGATGCT
     RBS          M  N  T  I  T  I  C  K  F  D  V  L  D  A
                                                              1800
GAACTTCTTTCGACAGTTGAGGGTGGATACTCTGGTAAGGATTGTTTAAAAGACATGGGA
 E  L  L  S  T  V  E  G  G  Y  S  G  K  D  C  L  K  D  M  G
                          ▲ ThmA                              1860
GGATATGCATTGGCAGGAGCTGGAAGTGGAGCTCTGTGGGGAGCTCCAGCAGGAGGTGTT
 G  Y  A  L  A  G  A  G  S  G  A  L  W  G  A  P  A  G  G  V
                                                              1920
GGAGCACTTCCAGGTGCATTTGTCGGAGCTCATGTTGGGGCAATTGCAGGAGGCTTTGCA
 G  A  L  P  G  A  F  V  G  A  H  V  G  A  I  A  G  G  F  A
                                                              1980
TGTATGGGTGGAATGATTGGTAATAAGTTTAACTAAGGAAGGAGTTTATATCATGAAGCA
 C  M  G  G  M  I  G  N  K  F  N Stop   RBS          M  K  Q
                                                              2040
GTATAATGGTTTTGAGGTTCTACATGAACTTGACTTAGCAAATGTAACTGGCGGTCAAAT

 Y  N  G  F  E  V  L  H  E  L  D  L  A  N  V  T  G  G  Q  I
                                                    ▲ ThmB
TAATTGGGGATCAGTTGTAGGACACTGTATAGGTGGAGCTATTATCGGAGGTGCATTTTC
 N  W  G  S  V  V  G  H  C  I  G  G  A  I  I  G  G  A  F  S
                                                              2160
AGGAGGTGCAGCGGCTGGAGTAGGATGCCTTGTTGGGAGCGGAAAGGCAATCATAAATGG
                                                RBS       OrfC
 G  G  A  A  A  G  V  G  C  L  V  G  S  G  K  A  I  I  N  G
                                                           M
                                                              2220
ATTATAAAAGTCTTTTATCGCTTTTATTATTCATAATTCCCCTTGTAGTTATACTAATCG
  L Stop
 D  Y  K  S  L  L  S  L  L  L  F  I  I  P  L  V  V  I  L  I
                                                              2280
GTCTTCGAAAGAATAATCAGAAACTAATCATTGCTGGGGTAGCTCCTCTTATCTATCTAT
 G  L  R  K  N  N  Q  K  L  I  I  A  G  V  A  P  L  I  Y  L
                                                              2340
TATGCAGTTATCTTTTAGACTGGATTTTTGATTGAGAAATTCGATTTTGGTCTATTTGTA
 L  C  S  Y  L  L  D  W  I  F  D Stop
GATTTTCAAAGTATGCTAAATGTTTTTAACCTCCCCAAAAATTATCAAGTCAAGTACAGC
AAAGTATTCATCTTGAACGGTTGAATAGTTATACAAAAGATATCTCATGATAAAAAACAG
ACATAGGAAATAAATTTGGAGGCTGATATTGATATGAAATTTTGATAAACATATTGAATT
                                                              2580
AAATACCTTCGTCTTGGAAGAAATTCAAGGAGGAAACCCAAATTGGGGGTTCAGTATTTG
                                  Rho independent Terminator
```

FIG. 4. **Nucleotide sequence and deduced polypeptide sequences of the Thermophilin 13 gene cluster.** The 960-bp DNA sequence localized 1600 bp downstream of the *Eco*RI site of the cloned 3.8-kb *Hin*dIII-*Eco*RI fragment is shown. The structural genes of Thermophilin 13 (*thmA* and *thmB*) together with their signal sequences (*underlined*) are depicted *below* the nucleotide sequence. The GG-processing sites are indicated by *bold triangles*. ORFC is a third peptide, encoded by the Thermophilin 13 operon. Putative promoter sequences, ribosome binding sites (*RBS*), start and stop codons, and rho-independent terminator sequences are indicated in *bold*.

TABLE III
Molecular weights (M_r) of the purified peptides deduced from those of their multiply charged ions determined by electrospray mass spectrometry

	$[M_r + 2H^+]/2$	$[M_r + 3H^+]/3$	$[M_r + 4H^+]/4$	$[M_r + 5H^+]/5$	M_r +/− 1
ThmA		1926	1445	1156	5776
		1926	1445	1156	5776
ThmA′		1931	1449	1159	5792
		1936	1453	1162	5808
ThmB	1956	1304			3910
ThmB′	1948	1298	974		3892

Genetic Organization of the Bacteriocin Operon—Amino-terminal sequences of ThmA and ThmB were determined (initial yield of 1150 and 1160 pmol, respectively). Oligonucleotide primers were designed on the basis of the amino-terminal sequence of ThmA (48 residues): YSGKD*X*LKDMGGYAL-AGAGSGAL*X*GAPAG*X*VGALPGAFVGAHVGAIAG. The sequences DMGGYA and HVGAIA were used to design the forward (For1) and six different reverse primers (Rev1–6), respectively. The expected 128-bp fragment was obtained in a

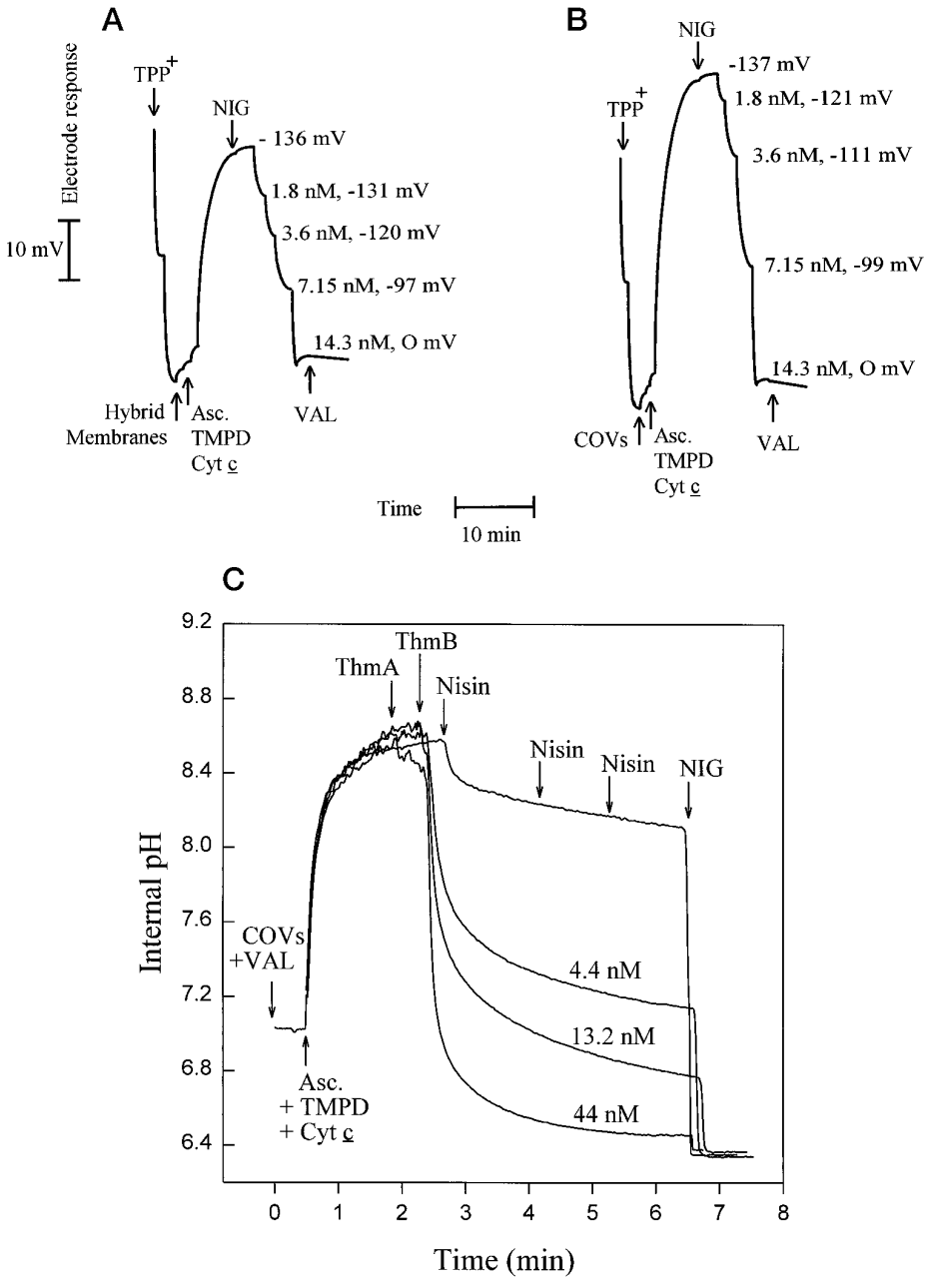

FIG. 5. ***In vitro* pore-forming activity of Thermophilin 13.** *A*, cytochrome *c* oxidase-containing proteoliposomes were fused to membrane vesicles of the sensitive SFi3 strain and used at a final lipid concentration of 0.2 mg/ml. TPP^+ (4 μM), ascorbate (*Asc*., 10 mM), TMPD (200 μM), cytochrome *c* (*Cyt c*, 10 μM), and nigericin (*NIG*, 100 nM) were added as indicated. Thermophilin 13 was added to final concentrations of 1.8, 3.6, 7.15, and 14.3 nM, and the obtained $\Delta\Psi$ values for each concentration are indicated. Valinomycin (*VAL*) was added to a final concentration of 200 nM. *B*, effect of Thermophilin 13 on the $\Delta\Psi$ in cytochrome *c* oxidase liposomes (*COVs*); conditions were the same as for *A*. *C*, effect of Thermophilin 13 on the internal pH in COVs. Experimental conditions were the same as in *B* except that pyranine (0.5 mM, internal concentration) was entrapped in the liposomes. The amounts of ThmA plus ThmB (4.4, 13.2, or 44 nM, each) are indicated. Nisin was added at final concentrations of 1.5, 3.0, and 4.5 μM, respectively (subsequent additions of 1.5 μM).

polymerase chain reaction with the primers For1 and Rev1 using SFi13 genomic DNA as template. The 128-bp probe hybridized to a 4.2-kb *Hin*dIII and a 3.8 *Hin*dIII-*Eco*RI fragment in the producer strain SFi13; these fragments were not detected by hybridization in the non-producer sensitive SFi3 strain. Subsequently, SFi13 chromosomal *Hin*dIII-*Eco*RI DNA fragments of 3.6–4 kb were ligated into pUC19, and the recombinant plasmids were transformed to *E. coli* BZ234. Colony hybridization identified 16 putative positive colonies, among a total of 250, containing a 3.7-kb *Hin*dIII-*Eco*RI insert that was sequenced and searched for open reading frames (ORFs) (Fig. 4). Two ORFs corresponding to ThmA and ThmB were found. Both peptides are synthesized with a signal sequence typical of lactic acid bacteria peptide bacteriocins, *i.e.* the processing site is preceded by a double glycine motif. The calculated molecular masses of ThmA and ThmB are 5776.7 and 3910.5 Da, respectively. The translation of ThmB is most likely coupled to that of a 52-amino acid hydrophobic peptide, termed ORFC; no homologue of ORFC was found in the data bases. Putative promoter elements, ribosome binding sites, and a rho-independent terminator structure were found in the regions flanking the ORFs (Fig. 4).

Chemical Modifications of ThmA and ThmB—Genetic analysis of ThmA and ThmB showed the presence of two cysteines in each peptide. Since Thermophilin 13 was fully active in the presence of 50 mM dithiothreitol, using the agar well assay, disulfide bonds are most likely not required for activity. The calculated average molecular masses of ThmA (5776.7 Da) and ThmB (3910.5) are consistent with the masses obtained after purification on Resource-RPC, *i.e.* 5776 ± 1 and 3910 ± 1 Da, respectively (Table III). However, a peak shoulder was noticed upon re-chromatography of ThmB on Resource-RPC (Fig. 1). MS analysis revealed a mass of 3892 Da in the shoulder (Table III), which is ~18 Da lower than that calculated from the translated nucleotide sequence of ThmB. This reduction in mass also occurred in the synthesized peptide ThmB upon prolonged storage but did not affect the enhancing properties of ThmB (purified or synthesized). Electrospray-MS also revealed

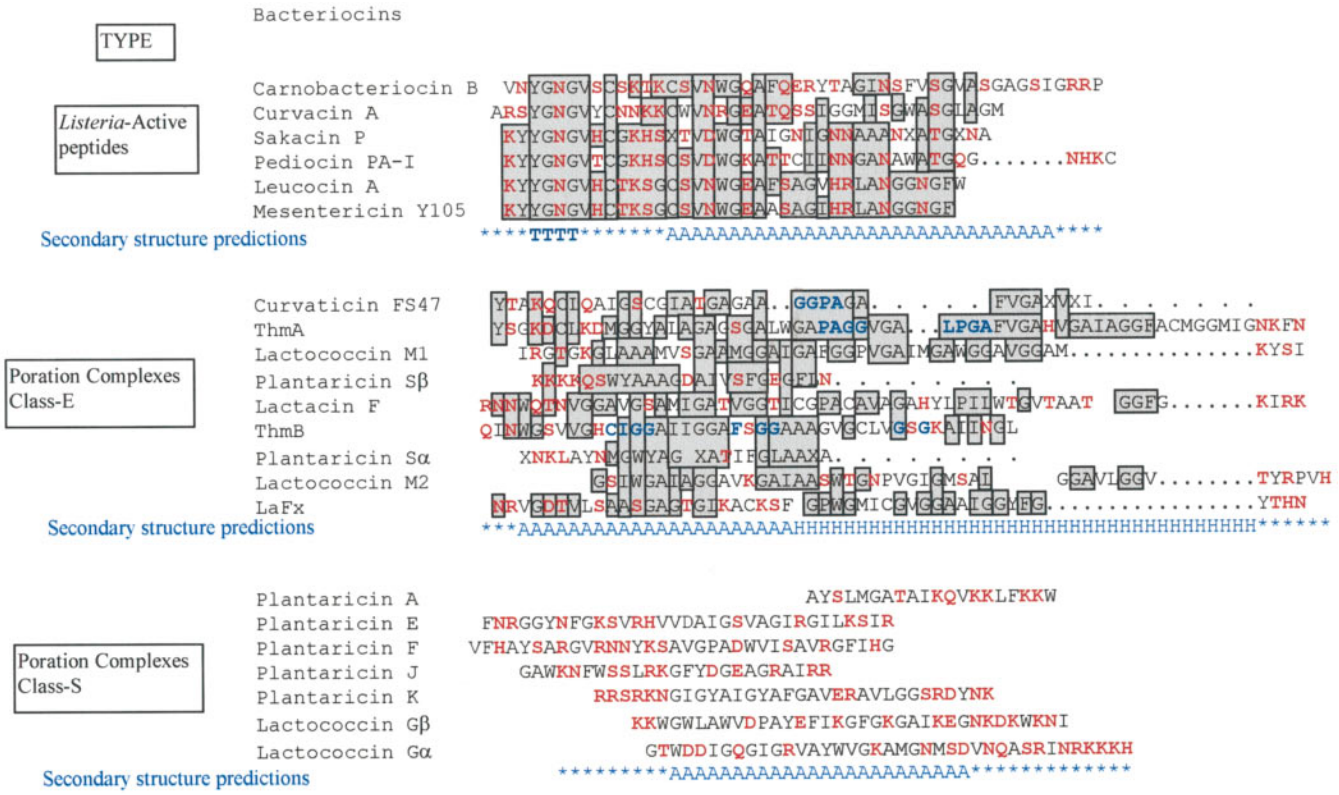

FIG. 6. **Sequence comparison of poration complexes and *Listeria*-active peptides.** Sequences were aligned by PileUp (GCG package). In addition to the *Listeria*-active peptides, the poration complex bacteriocins are classified as Class E (for enhancing) and Class S (for synergy). Hydrophilic residues are shown in *red*; *shaded boxes* symbolize identity within a family. Tetrapeptides predicted to be in a β-turn conformation (p(t) $\times 10^{-4} > 1.5$ (14, 15)) are printed in *dark blue*. Consensus in secondary structures predictions are indicated in *blue*, for each class of peptides: *, *A*, *T*, and *H* refer to hydrophilic (non-transmembrane segment), amphipathic α-helix, hairpin turn, and hydrophobic (α-helix or β-sheet) transmembrane segment, respectively.

chemical modifications in ThmA; the modified product was termed ThmA′. Comparison of the MS spectra of ThmA and ThmA′ shows two additional peaks for each multiply protonated species, leading to triplets for the ions with 3, 4, and 5 charges ($M_r + 3H^+$, $M_r + 4H^+$, or $M_r + 5H^+$) (Table III). These data indicate that ThmA′ has gained one or two times 16 Da (average of 3×5, 4×4, and 5×3 Da), which corresponds to the gain of one or two oxygen atoms and suggests that ThmA becomes oxidized upon storage. Although ThmA′ was as active as ThmA (Fig. 2, *right panel, top part*), the activity of ThmA′ was no longer enhanced by ThmB (Fig. 2, *right panel, lower part*). Oxidation of ThmA to ThmA′ could be prevented by storage at −20 °C. Neither ThmA nor ThmB contain post-translational modifications common to those of lantibiotics or cystibiotics like Pediocin PA-I (6). In the experiments described below, Thermophilin 13 refers to an equimolar ratio between ThmA (non-oxidized) and ThmB (both forms).

In Vitro Pore-forming Activity of Thermophilin 13—The poration complex bacteriocins have a narrow host range specificity and require a "receptor" for pore-forming activity (7). The poration complex bacteriocins function in membrane vesicles derived from sensitive species but not in (proteo)liposomes, with the exception of Lactococcin G, which is only active in whole cells (24). Thermophilin 13, on the other hand, affects a large variety of organisms (Table II), suggesting that it might not require a specific factor to exert its bactericidal activity. To investigate whether Thermophilin 13 exerted a pore-forming activity in target membranes, we studied the ability of the bacteriocin to dissipate the membrane potential (ΔΨ) in cytochrome *c* oxidase containing liposomes (COVs) fused with membranes of the sensitive *S. thermophilus* strain SFi3. In the presence of the electron donor system potassium ascorbate/TMPD/cytochrome *c*, a ΔΨ (inside negative) and a ΔpH (inside alkaline) was generated. To clamp the ΔpH to 0, nigericin was present in the measurements of ΔΨ. Increasing amounts of purified Thermophilin 13 dissipated the ΔΨ progressively (Fig. 5*A*). To establish the influence of membrane proteins and/or lipids of the target organism on this putative pore-forming activity, the bacteriocin was also tested on COVs solely composed of *E. coli* lipids plus egg phosphatidylcholine at a molar ratio of 3 to 1. Thermophilin 13 also dissipated the ΔΨ in COVs (Fig. 5*B*). ThmA alone had little effect, and ThmB alone had no effect on the ΔΨ both in the hybrid membranes and COVs (data not shown). To investigate the activity of Thermophilin 13 in the absence of a membrane potential, the effect of Thermophilin 13 on the pH gradient in COVs was analyzed. Valinomycin was used to keep ΔΨ at zero, and the COVs were loaded with the pH indicator pyranine. It appeared that Thermophilin 13 was able to dissipate the ΔpH at nanomolar concentrations, whereas similar concentrations of ThmA and ThmB alone had virtually no effect (Fig. 5*C*). Nisin had only little effect on the ΔpH in the COVs at micromolar concentrations.

Effect of Thermophilin 13 on the Cytochrome c Oxidase Activity—It was possible that Thermophilin 13 dissipated the ΔΨ and ΔpH by inhibiting cytochrome *c* oxidase activity. Up to a concentration of 100 nM, we observed no effect on cytochrome *c* oxidase activity reconstituted in liposomes; the measurements were performed in the presence of nigericin plus valinomycin to prevent increases in oxidase activity due to respiratory control. Overall, these results suggest that Thermophilin 13 is able to dissipate ΔΨ in the absence of ΔpH and to dissipate ΔpH in the absence of ΔΨ, without the need for a specific proteinaceous receptor or lipids from a sensitive host strain.

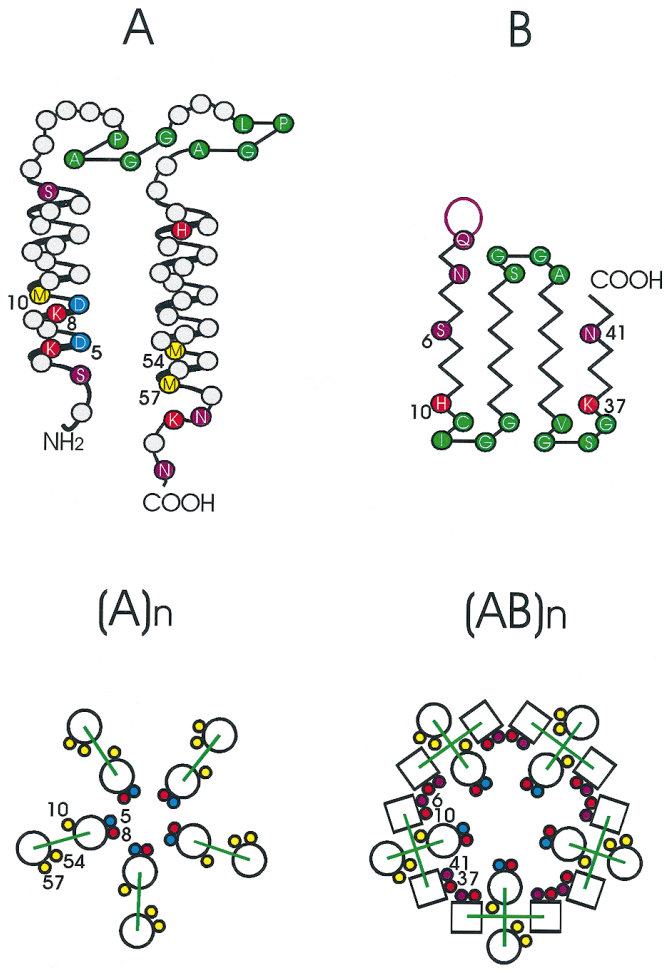

FIG. 7. **Structure models of the poration complex formed by Thermophilin 13.** Secondary structure models of ThmA (*A*), ThmB (*B*), and top view models of the pores formed by ThmA (*A*)*n*, and ThmA plus ThmB (*AB*)*n* (*i.e.* Thermophilin 13) are shown. Cationic residues (*K*, *R*, and *H*) are represented in *red*; anionic residues (*D* and *E*) are indicated in *blue*, hydrophilic amino acids (*S*, *T*, *N*, and *Q*) are depicted in *purple*. Methionine residues (Met^{10}, Met^{54}, and Met^{57}) likely to form methoxides are indicated in *yellow*. Tetrapeptides predicted to form β-turns of $p(t) \times 10^{-4} > 1.5$ (14, 15) are depicted in *green*. In the top view models, helices are represented by *circles*, and two β-strands are symbolized by a *square*.

DISCUSSION

In this paper, we describe a novel antimicrobial compound, Thermophilin 13, which differs from other known bacteriocins in its structural and/or functional properties. (i) Thermophilin 13 exerts an activity in COVs which so far has only been observed for bacteriocins of the lantibiotic type; (ii) Thermophilin 13 has antilisterial activity but lacks the YGNGV-C motif, typical of the *Listeria*-active peptides; (iii) Thermophilin 13 forms a poration complex but in contrast to other "two-component bacteriocins" it has a broad host range activity.

The experiments in COVs, prepared from *E. coli*/egg phosphatidylcholine lipids (Fig. 5), indicate that Thermophilin 13 does not need a specific component (proteinaceous or lipid) in the membrane for activity. It should be stressed that, although a receptor has not yet been identified for any lactic acid bacterial bacteriocin, the "non-lantibiotics" require an "additional factor" in the target membrane to exert pore-forming activity (6, 25). In fact, only lantibiotics, and bacteriocins thought to contain lanthionines (*e.g.* Plantaricin C), have so far been shown to exert pore-forming activity in COVs (2, 26, 27). Furthermore, and in contrast to the lantibiotics (2), Thermophilin 13 does not require a threshold membrane potential to dissipate the pH gradient (Fig. 5*C*) or a threshold pH gradient to dissipate the membrane potential (Fig. 5*B*).

The experiments also established that Thermophilin 13 forms a poration complex that is composed of an equimolar ratio of the two peptides ThmA and ThmB (Fig. 3*A*). ThmA alone has antibacterial activity against *S. thermophilus*, *C. botulinum*, *L. monocytogenes*, and *B. cereus*, which is enhanced ~40-fold when an equal amount of ThmB is present. ThmB, by itself, is not bactericidal, and an excess of this peptide inhibits the activity of ThmA (Fig. 3*D*), possibly because it destabilizes the pore leading to dysfunctional oligomeric structures. By comparison, Lactacin F (28), and Plantaricin S (29) are also composed of an active and enhancing peptide, whereas the two peptides of Lactococcin G have virtually no activity when tested separately, *i.e.* 5×10^5 times less than when used in combination (24). These considerations have led us to subdivide the poration complex bacteriocins into two classes: type E, for Enhancing, *i.e.* when one of the peptides only functions as an enhancer as for Thermophilin 13, Lactacin F and Plantaricin S, and type S, for Synergy, *i.e.* when activity is believed to require the combination of both peptides, *e.g.* Lactococcin G (24) and Plantaricin A (30). Interestingly, this functional classification is substantiated by similarities in primary sequence and predicted secondary structure of the peptides (Fig. 6). Type E peptides are characterized by several G(A/G)G repeats and are predicted to form an amino-terminal amphipathic α-helix followed by a hydrophobic anchor. On the basis of these criteria, Lactococcin M (31) and Curvaticin FS47 (32) were classified as type E peptides (Fig. 6). The amino-terminal amphipathic α-helix of ThmA could be stabilized by a salt bridge between Asp^5 and Lys^8 and by hydrogen bonding between the amino- and carboxyl-terminal parts (Fig. 7*A*). By contrast to other type E peptides, three β-turns are predicted in the sequence of ThmB, at distances that allow the intervening sequences to span the membrane as antiparallel β-sheets (Fig. 6); the presence of helix-breaking residues (serines and asparagines) and the prediction of two amphipathic strands further support a β-sheet conformation for ThmB. Type S peptides are characterized by an amphipathic α-helix composed of charged residues at one face of the helix and highly hydrophobic residues at the opposite face (Fig. 6). On the basis of structural similarities with Lactococcin G (8) and Plantaricin A (30), Plantaricin E, F, J, and K (33) have been placed into class S peptides (Fig. 6) even though experimental evidence that these bacteriocins form similar poration complexes is lacking.

High probabilities of hairpin turns are not only found in ThmB but also in ThmA, in the *Listeria*-active peptides (see Ref. 6 for review), and downstream of the amphipathic α-helix of the partially sequenced peptide Curvaticin FS47 (32) (Fig. 6). Since Thermophilin 13 and Curvaticin FS47 have been shown to exert antilisterial activity, we speculate that the hairpin turn might be one of the critical elements of the GG-processed peptides to exert antilisterial activity. In this respect, it is worth emphasizing that the membranes of *Listeria* species differ significantly from that of lactic acid bacteria, not only in lipid conjugates (*e.g.* lipoteichoic acids), apolar lipids (*e.g.* isoprenoid quinones), but also in fatty acids (34).

The sequence analysis has been used to derive a structure model for the poration complexes formed by ThmA (plus ThmB). The intrinsic activity observed for ThmA and ThmA′ can be explained by oligomerization of the peptide to form the structure ((*A*)*n*) (Fig. 7). The MS analysis of ThmA′ strongly suggests oxidation of methionines to methoxides (Met^{10}, Met^{54}, and/or Met^{57}; *yellow circles* in Fig. 7), which would not dramatically influence the pore-structure as shown in Fig. 7, (*A*)*n*, but would disturb the interactions between ThmA and ThmB (Fig.

7 (*AB*)*n*). The enhancing properties of ThmB can be explained by stabilization of ThmA through subunit/subunit interactions and/or participation of ThmB to the pore hydrophilicity (Ser^6, His^{10}, Asn^{41}, and Lys^{37} in Fig. 7). The proposed model of the poration complex formed by ThmA and ThmB reminds us of the acetylcholine receptor channel (35, 36). Finally, The spontaneous loss of 18 Da in ThmB is consistent with the cyclization of the amino-terminal glutamine.

In conclusion, Thermophilin 13 shares functional properties of lantibiotics and structural properties of, what we propose to name, type E poration complexes. The prediction of β-turns in putative loop regions of ThmA, YGNGV-C peptides, and Curvaticin FS47 suggests a structural basis for the interaction of these peptides with the membranes of *Listeria* species. Our model of the poration complex is consistent with the difference in enhancing properties of ThmB on ThmA and ThmA′. Finally, the ionophoric activity of Thermophilin 13 in the absence of a membrane potential, and without the need for a receptor, makes it an extremely potent compound that differs in its mode of action from Nisin and other bacteriocins described so far.

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